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14. ABSTRACT

The objective of this project is to discover and characterize molecules that inhibit breast cancer cell proliferation by maintaining activity of the retinoblastoma protein (Rb). Rb is inactivated to drive proliferation in normal and cancer cells by phosphorylation, which dissociates the E2F transcription factor from Rb. Our goal is to find and characterize molecules that stabilize the complex between phosphorylated Rb and E2F.

In this first year of the project period, we successfully optimized a fluorescence polarization assay for Rb-E2F binding suitable for high-throughput screening. Together with scientists at the Sanford Burnham Institute, we then screened 350,000 compounds for activity stabilizing the complex. 236 hit compounds were found in the primary screen, of which 108 compounds repeated in a dose response assay. We are now in position to follow these lead compounds in secondary assays and characterize their mechanism of action.

15. SUBJECT TERMS
cell cycle, Retinoblastoma protein, E2F transcription factor, high throughput screen, drug discovery

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1.0 INTRODUCTION

The Rb pathway connects environmental and intracellular growth signals to the cell cycle machinery that drives cell division. Inactivation of Rb pathway function is found in most human cancers, including breast cancer. Inhibition of cell proliferation by Rb is linked to its direct binding of E2F transcription factors and repression of E2F activity. Rb inactivation occurs upon Cdk phosphorylation, which induces E2F release and activation of S phase genes. *Our overarching hypothesis is that we can modulate Rb activity directly with small molecules that inhibit or stabilize its association with E2F and thus control cell proliferation.* This project aims to identify such molecules with high-throughput screening, to validate hits in secondary and cellular assays, and to characterize the mechanism of lead compound interaction with Rb. The first year of the project was aimed specifically at screening the large small molecule library of ~320,000 compounds at the Conrad Prebys Center for Chemical Genomics (CPCCG, at Sanford Burnham Institute) to identify such compounds, confirm their activity, and remove artifact compounds as a starting point for future optimization and detailed chemical biological studies. We successfully identified 108 reproducible hits and are now moving forward with further validation.

2.0 KEYWORDS

Retinoblastoma (Rb) pathway, E2F transcription factor, cancer, cell cycle inhibition, activation, modulation, inhibition, high throughput screening, structure activity relationships (SAR), hit validation, PAINS.

3.0 ACCOMPLISHMENTS

Summary:

In collaboration with the CPCCG, we implemented a fully-automated 1536-well fluorescence polarization-based primary screen, first optimized in the Rubin laboratory, to detect molecules that increase E2F binding to Rb. We used this assay to screen CPCCG's large (~320,000 compounds) chemical library, and identified and validated novel chemical scaffolds with *in vitro* potency. In the first year of the project period, we achieved the milestone of identifying 108 verified hit compounds and nascent SAR to select chemotypes for efficacy and selectivity validation.

Goal:

Perform high throughput florescence polarization screen and reproduce hits with dose-response assay.

Detailed Accomplishments:

Task 1: Optimize screen for 1536-well format

The Rubin laboratory provided protocols and a test lot of reagents for the 1536-well formatted fluorescence polarization (FP) assay. The assay was originally developed in the Rubin laboratory for 384-well plates and required testing and potential optimization for the higher-throughput format. The assay comprises a peptide corresponding to the E2F transactivation domain (human E2F2 amino acids 409-428) synthesized with a tetramethylrhodamine dye (TMR) at its N-terminus (E2F$_{TMR}$). This peptide is incubated with a recombinant Rb$_{NP}$ construct with or without compounds. Phosphorylation of this Rb construct recapitulates the loss of E2F$_{TD}$ binding, which is necessary and sufficient for Rb inactivation in

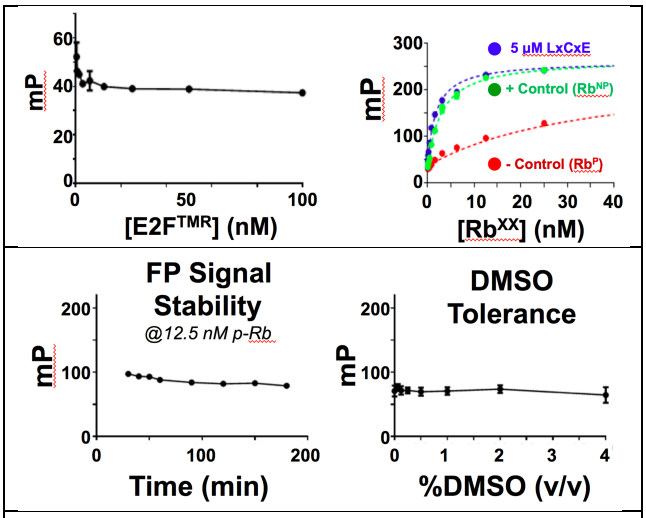


Figure 1. Screen optimization in high-throughput 1536-well format.

cells. CPCCG completed validation and implementation of the fully automated 1536-well assay, conducted a pilot HTS and reviewed data, and approved go-ahead for full screening in a 2/20/15 teleconference with Dr. Rubin.

1536-well Assay validation results: The CPCCG team recapitulated the binding pharmacology of the assay FP signal (mP) with respect to E2F$_{TMR}$, where equivalence (signal plateau) was obtained at ~12.5 nM consistent with a 1:1 E2F$_{TMR}$: Rb$_{NP}$ complex (Figure 1, top left). They also recapitulated the reduction of binding upon phosphorylation of Rb$_{NP}$(□) to Rb$_{P}$(□) and increase of binding with the positive LxCxE(□) peptide control, which restores Rb$_{P}$ to tight binding (Figure 1, top right). Additionally, the signal was stable up to 3 hr and the assay was very tolerant up to 4% DMSO (v/v) (Figure 1, bottom).

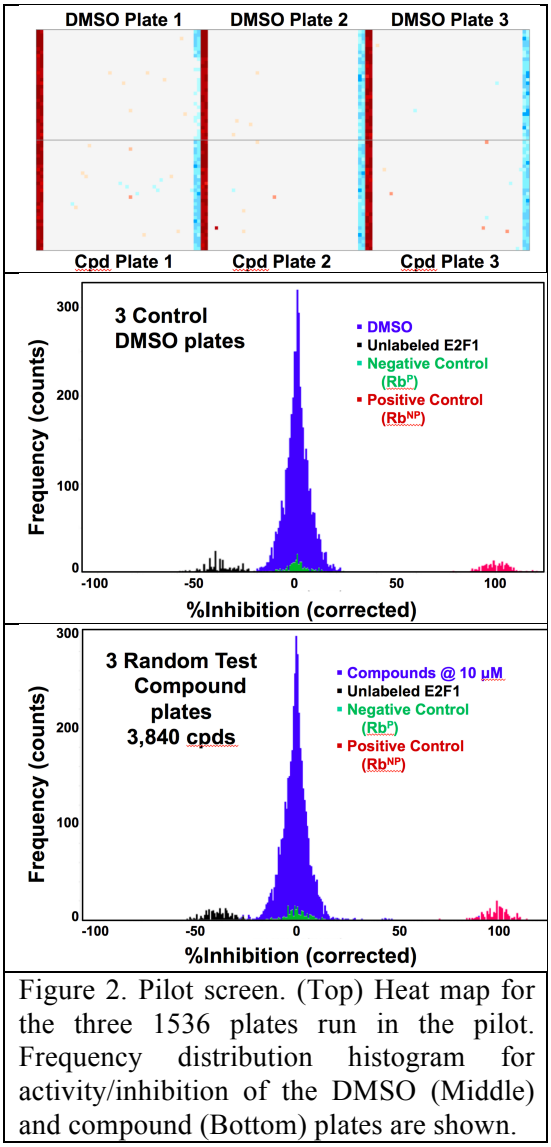


Figure 2. Pilot screen. (Top) Heat map for the three 1536 plates run in the pilot. Frequency distribution histogram for activity/inhibition of the DMSO (Middle) and compound (Bottom) plates are shown.

Based on these results, the CPCCG team performed a pilot HTS on 3 compound plates & 3 DMSO plates (2 µL+ 2 µL assay volume) with 10 µM final compound concentration in black, 1536-well, solid bottom Corning #3724. No hits were obtained at low thresholds for DMSO plates (Figure 2, top and middle), giving us confidence in hit calling for this challenging protein-protein interaction target class.

The frequency distribution for DMSO controls are "normal" Gaussian (Figure 2, middle) and consistent with the complete lack of false hits in the plate "heat maps" (Figure 2, top). The frequency distribution for the pilot screen of 3,840 compounds shows a Gaussian centroid with a few "spikes" or hits near 45% inhibition, which is consistent with the few observed hits on the plate heat maps (Figure 2, top and bottom). We note the apparent negative %inhibition for the unlabeled E2F1 is due to the effective dilution of specific activity of the E2F$_{TMR}$ at high unlabeled E2F, which leads to a lower baseline absolute fluorescence signal, for which even a ratiometric FP calculation cannot completely compensate. However, these data are consistent with a validated FP assay for RB$_{NP}$ and E2F$_{TMR}$ binding.

Task 2: Perform screen with CPCCG compound library sets

Parameter	Value
Positive Control (mP)	195.7 ± 8.6
Negative Control (mP)	52.6 ± 6.9
Signal to noise	17.10 ± 1.91
Z'	0.67 ± 0.03
Hits (>30% activity)	236
Hit Rate %	0.07

Based on assay validation and implementation, we committed to completing scale-up of a production lot of protein reagents (~100 µL of 100 µM E2F$_{TMR}$, ~50 µL of 80 µM Rb$_{NP}$, and 200 µL). Reagents were generated in the Rubin laboratory, quality control was performed with an FP assay at UCSC, and the proteins were sent to the CPCCG. CPCCG completed the full HTS campaign on 5/15/15, testing 320,000 compounds. Assay performance during the HTS campaign

was robust as shown in the Table above, with no individual plate running with less than 0.65 Z'. Based the superior performance we set the threshold at 30% inhibition as the hit rate. The hit rate at this threshold (0.07%) was low as expected for this difficult target class of a protein-protein interaction.

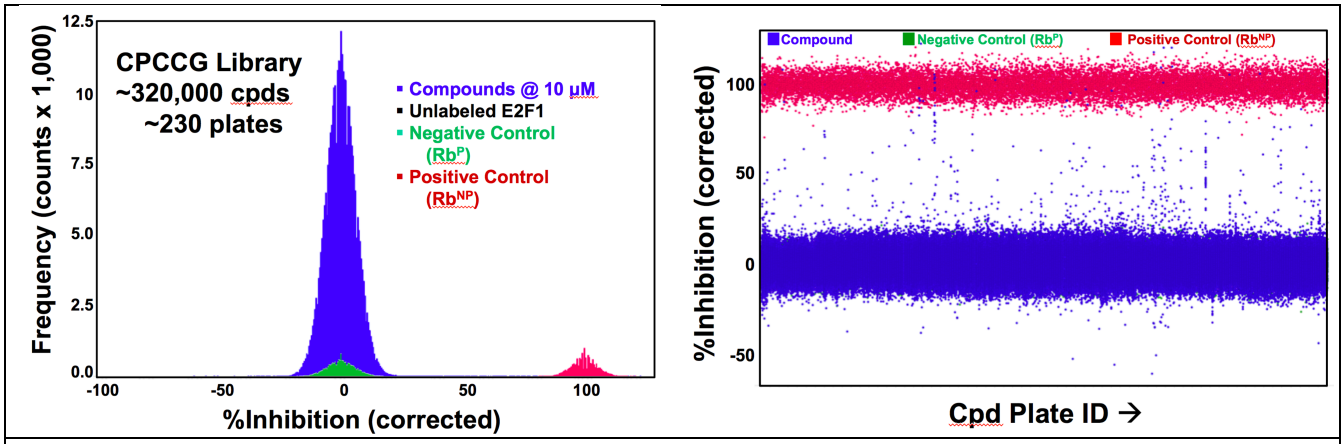
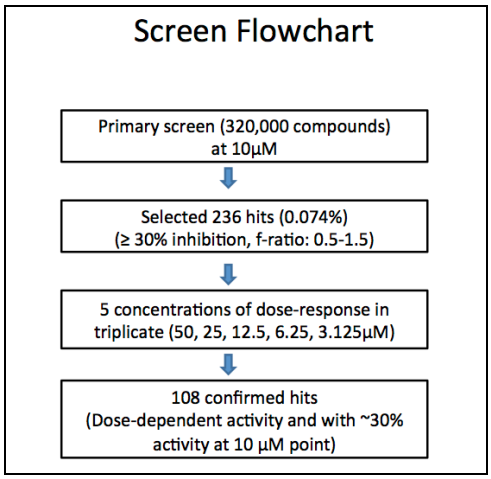


Figure 3. Results from 320,000 compound screen at the CPCCG.

The frequency distribution and the scattergram for the screen (Figure 3) are consistent with a robust normally distributed centroid of zero activity, with clear hits. At the threshold of >30% activity, we selected 236 compounds, which had an F-ratio between 0.5 – 1.5 (an indication that the compound is not fluorescent or absorption artifact), as primary hits.

Task 3: Cherry-pick hits and rescreen with dose-response in triplicate

Because of the relatively low number of primary hits obtained, we forewent the process of triplicate cherry picking for a confirmation at the single original screening concentration, but rather went straightaway into triplicate 5-point dose-response analysis (50, 25, 12.5, 6.25, 3.125 µM final) using the CPCCG "direct dilution" paradigm with their Labcyte Echo 555 acoustic drop ejection technology, where every point on a dilution curve represents a true independent (rather than serial) dispensing event. With this, we confirmed 108 compounds as giving a dose-response curve with at least 30% inhibition at 10 µM. The flowchart of hit progression is shown on the right. Ultimately, 20 confirmed hits showed an estimated IC50 <10µM.



Task 4:Filter hits with PANS and select compounds to move forward with validation.

CPCCG's Cheminformatics team clustered the 108 confirmed hits by scaffold class, sorted them by potency, and also flagged PAINS (Pan Assay Interfering Compounds, which are common false positives). This analysis resulted in 18 scaffold groups (clusters containing 2 – 9 exemplars) and 38 singletons (only one exemplar in the group) to account for all 108 confirmed hits. We also called out the most potent exemplar of each cluster, though we deprioritized those compounds with dose response curves, which did not fit well. A few of the clusters showed a span of 5-fold potency, which suggests emergent SAR and provides confidence that these are bonafide tractable hits.

Training Opportunities: Nothing to report
Dissemination of Results: Nothing to report

Future plans:
We are now in the process of ordering hit compounds as fresh powder for validation and further study in secondary assays. We will look at activity in influencing the cell cycle and proliferation of breast cancer cells. We will also make biophysical measurements of compound affinity for Rb and determine how E2F binding is modulated.

4.0 IMPACT

Through a robust HTS interrogation of 320,000 compounds, we have confirmed and validated from master stock solutions of these compounds 108 potential compounds representing distinct chemical scaffolds. Several hits have single digit micromolar potency and emergent SAR. These hits may provide bonafide starting points for further elucidation of SAR by catalog to derive bonafide, chemically tractable, potent and selective small molecule inhibitors/modulators of the E2F-Rb interaction.

5.0 CHANGES / PROBLEMS

We encountered no technical problems. The hit rate was predictably low for this protein-protein interaction target, though the assay robustness allowed a fairly low threshold. Given the relatively low number of primary hits, we changed our experimental plan to forego the cherry picking into single concentration, but rather went directly into dose-response analysis.

No other significant changes to protocols to report.

6.0 PRODUCTS

Nothing to report.

7.0 PARTICIPANTS

Name:	Dr. Seth Rubin	Dr. Thomas Chung	Cameron Pye
Project Role:	PI	Collaborator	Graduate Student Researcher
Person Months:	1	1	6
Contribution to project:	Oversee project, data interpretation, and communication of results	Conduct screening at CPCCG with staff	Reagent production and assay development at UCSC

8.0 SPECIAL REPORTING REQUIREMENTS

None

9.0 APPENDICES

None